Inheritance of nrDNA in artificial hybrids of *Hesperocyparis arizonica* x *H. macrocarpa*

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ABSTRACT

Sequencing nrDNA of parents (*Hesperocyparis arizonica, H. macrocarpa)* found their nrDNA differ at 8 sites. Analysis of 18 artificial hybrids, revealed each of the hybrids had nrDNA that was heterozygous at each of the 8 sites. However, the peak ratios in the chromatograms were not 1:1 as expected, but varied from 1:1 to 3:1, being more like *H. arizonica*. PCO of the variation in the peak heights revealed four groups of hybrids that might be associated with chromosome inheritance. However, PCO clearly distinguished the parents and the hybrids. nrDNA, if appropriately coded, can be utilized in multivariate methods for the detection of hybrids. Due to concerted evolution, nrDNA may underestimate the degree of introgression. Analysis of petN-psbM (cp DNA) confirmed that all the hybrids inherited the cp genome from their pollen-parent (*H. arizonica*) confirming cp genome inheritance via pollen in *Hesperocyparis* (Cupressaceae). Published on-line **www.phytologia.org** *Phytologia 98(4): 277-283 (Oct. 6, 2016).* ISSN 030319430.

KEY WORDS: *Hesperocyparis arizonica, H. macrocarpa,* Cupressaceae, hybrids, nrDNA, petN-psbM, inheritance of cp genome via pollen.

Sequencing of nrDNA spacer regions has been an important source of phylogenetic information in plant systematics for several years. The conserved nature of the multi-copy nrDNA (thousands of copies per cell) might be due to concerted evolution (Liao, 1999). Liao (1999) argues that because rRNAs are structural molecules, multiple gene copies are necessary to supply the demand for ribosomal subunits in the cell. Because these sub-units function only when assembled into a large complex, homogeneity of rRNAs is critical for regular, functional ribosome assembly and translation to function normally. Liao (1999) concludes that "a possible biological function of concerted evolution is to maintain homogeneous gene copies in a family so that homogeneous transcripts can be produced." However, concerted evolution is thought to be a slow process over numerous generations. Hybrids would seem likely to be heterozygous for both parents nrDNA. Thus, nrDNA (ITS) is often used for the analysis of hybridization. Recently, Adams (2015a,b) found that nrDNA detected 15 hybrids, whereas, maldehy, a single copy nuclear gene (SCN), detected 25 hybrids. nrDNA appeared more often to be the same as one of the parents, whereas the SCN gene (maldehy) was heterozygous, indicating the plant(s) were of hybrid origin.

Chaing et al. (2001) found that in the artificial hybrids between *Begonia aptera* (pollen) and *B. formosana* (maternal), nrDNA was predominantly that of the maternal parent, *B. formosana* (diamonds, Fig. 1). Volkov et al. (1999) reported that one of the parental nrDNAs was eliminated in the allopolyploid genome of cultivated tobacco. Fukuoka et al. (1994) found that the nrDNA in γ-ray irradiated tetraploid rice was homogenized in a short time.

Aguilar et al. (1999) made artificial hybrids between *Armeria villosa* ssp. *longiaristata* and *A. colorata*, then examined the inheritance of nrDNA in F_1 and F_2 generations. They found the expected

additive pattern in polymorphisms for five of the six variable sites in F_1 plants. However, in the F_2 generation, there was a bias towards one parent (*A. colorata*). Backcrosses showed homogenization of five of the polymorphic sites to the recurrent parent.

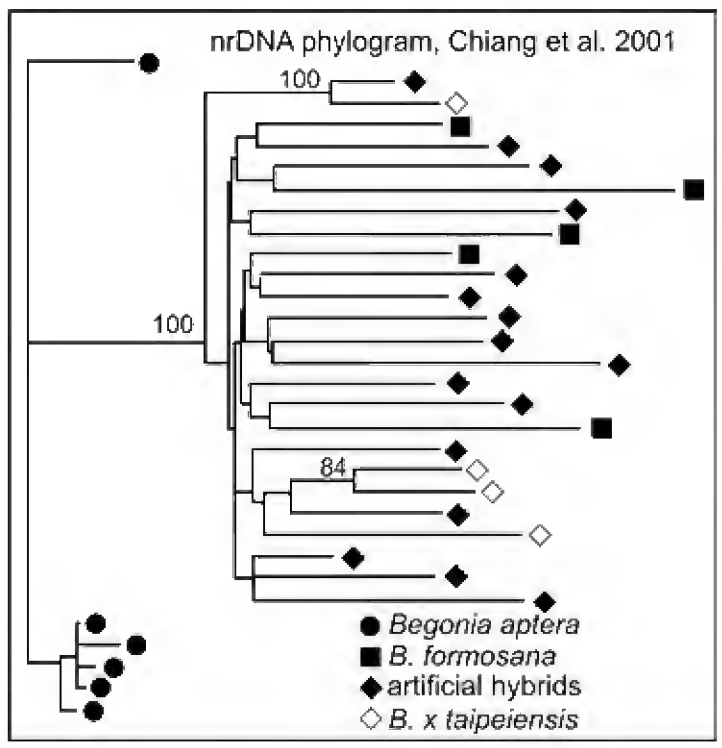

Figure 1. Phylogram based on nrDNA for *Begonia* and hybrids (adapted from Chiang et al. 2001). Notice the grouping of the hybrids (triangles, nrDNA) with the maternal parent, *B. formosana* (shaded squares), rather than with the pollen (paternal) parent *(B. aptera*, shaded circles).

Okuyama et al. (2005) examined introgression in *Mitella* using nrDNA ITS and ETS, and cpDNA and found that cpDNA revealed the most introgression, ITS regions showed a moderate amount of introgression and the ETS region gave no evidence of introgression. They concluded that non-uniform concerted evolution between the ETS region and ITS regions may explain these different patterns of introgression.

Adams and Matsumoto (2016) sequenced nrDNA of *Cryptomeria japonica* D. Don (Sugi) cv. *Haara* and cv. *Kumotooshi* (Kumo) and found 3 variable sites at positions 154, 468 and 505. In Kumo, each of these three positions was heterozygous, indicating that Kumo is a hybrid between Haara x *C. japonica*. If so, then the progeny of this cross would be backcrosses [(Haara x *C. japonica*) x Haara].

Table 1. Variable sites in the nrDNA sequence for *Cryptomeria japonica* cv. *Haara, C. japonica* cv. *Kumotooshi* and their hybrids. The ratios of bases in parenthesis ( ) were obtained by measurements of the peak sizes on the chromatogram. NA = not available.

					petN-psbM (from pollen)		
	site 154	site 468	site 505	nrDNA type	site 145	site 146	cp type
Haara	C	T	G	Haara	A	T	Haara
Kumo	C/G (1:0.7)	T/C (0.64:1.0)	G/A (0.76:1)	Haara x Kumo	C	G	Kumo
progeny (backcrosses to Haara?):					Pollen parent of progeny		
14519	C/G (1:0.7)	T/C (1:1)	G/A (0.7:1)	Haara x Kumo	C	G	Kumo
14520	C/G (1:0.7)	T/C (0.46:1)	G/A (0.58:1)	Haara x Kumo	C	G	Kumo
14521	C/G (1:0.2)	T/C (1:0.5)	NA	Haara x Kumo	C	G	Kumo
14522	C	T	NA	Haara	C	G	Kumo
14523	C	T	NA	Haara	C	G	Kumo
14524	C	T	NA	Haara	C	G	Kumo
14525	C	T	NA	Haara	C	G	Kumo

Three of the seven progeny (BC) had nrDNA very similar to the (Haara x Kumo) parent, with some variation in the ratio of bases (Table 1). 14519 showed a small shift in frequency toward the recurrent parent (Haara) at position 468 (1:1 vs. 0.64:1 in Kumo). 14620 showed a shift in frequency toward Haara at position 468. 14621 shifts toward Haara at positions 154 and 468. Overall, all 3 of these progeny showed some shift in frequencies toward the recurrent parent (Haara), as one might expect from backcrossing.

The nrDNAs of four progeny (BC) (Table 1) were the same as the recurrent parent Haara. Positions 154 and 468 showed a complete shift in frequencies to the recurrent parent Haara by this single backcrossing event. This suggests that perhaps, nrDNA can rather quickly revert to the pattern of one of the parents in a backcrossing event by concerted evolution.

In the Cupressaceae, breeding programs are rare, so the existence of parents and artificial (verified) hybrids is an important resource for studies on inheritance. Scion Research Institute, Rotorua, New Zealand has a breeding program that involves crossing *Cupressus* and *Hesperocyparis* species. Sequencing nrDNA of *H. arizonica* (2003.017) and *H. macrocarpa* (896.752) found they differ at 8 sites and were each monomorphic in each taxon for each of the 8 sites. The breeding program afforded an unusual opportunity to examine the inheritance of nrDNA in hybrids in the Cupressaceae. As far as known, this report will be the second on the inheritance of nrDNA in the Cupressaceae (or in conifers). The purpose of this paper is to report on the inheritance of nrDNA in artificial hybrids of *H. arizonica* x *H. macrocarpa*.

MATERIALS AND METHODS

Plant material: Crosses were made at the Scion Research Institute, Rotorua, New Zealand using pollen of *H. arizonica* (2003.017) onto receptive seed cones of *H. macrocarpa* (896.752). Seedlings were obtained and greenhouse grown to 50-80 cm, then field planted. Leaf samples were taken after approximately one year in the field (plants about 1 m tall). Parents: *Adams 14854 H. arizonica* (2003.017), *Adams 14858 H. macrocarpa* (896.752), (leaves in silica gel)
Eighteen (18) Hybrids (leaves in silica gel) (lab accession #): *Adams 14914 - Adams 14931.*

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20° C until the DNA was extracted. DNA was extracted from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions.
Amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (petN-psbM) or K (nrDNA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used), 1.8 µM each primer. See Adams, Bartel and Price (2009) for the ITS and petN-psbM primers utilized. The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. Sequences for both strands were edited and a consensus sequence was produced using Chromas, version 2.31 (Technelysium Pty Ltd.).

RESULTS AND DISCUSSION

As mentioned in the introduction, sequencing nrDNA of *H. arizonica* (2003.017) and *H. macrocarpa* (896.752) resulted in 1249 bp with differences at 8 sites. Each of these 8 sites were monomorphic in each parent (Table 2). Sequencing nrDNA of the 18 hybrids revealed every hybrid was polymorphic at each of the 8 sites (Table 2). In contrast to the theoretical ratio of 1:1, none of the hybrids had exactly that ratio for the 8 sites (Table 2). However, 3 of the hybrids (14915, 14920, 14931, in bold,

Table 2. Variable sites in hybrids between *H. arizonica* (14854, 2003.017) x *H. macrocarpa* (14858, 896.752) cross which differ at 8 sites. nrDNA (ITS) numbering is from the 5' end. Chromatogram peak heights are ratio of peaks expresses as arizonica bp/ macrocarpa bp. (i.e. for 124, C:T 2:1 = 2.0; C:T 60:40 = 1.5, etc.). na = not available. petN sequence pattern (right-most column) shows the paternal (pollen) parent of the hybrid. Hybrids are grouped by similarities in boldface, italics, normal, and the second boldface group.

	124	177	295	313	370	516	775	1107	petN seq.
arizonica	C	C	T	C	G	C	G	A	*arizonica*
macrocarpa	T	G	C	T	T	T	A	G	*macrocarpa*
	C/T	C/G	T/C	C/T	G/T	C/T	G/A	A/G	ratios
theoretical F1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	
14915 group A	**1.0**	**1.5**	**2.0**	**1.0**	**3.0**	**1.0**	**1.0**	**1.0**	*arizonica*
14920	**1.0**	**2.0**	**1.0**	**1.0**	**2.0**	**0.5**	**1.0**	**1.0**	*arizonica*
14931	**1.0**	**1.5**	**1.2**	**1.0**	**2.0**	**1.0**	**1.0**	**1.5**	*arizonica*
14916 group B	*1.5*	*1.0*	*1.2*	*1.5*	*1.5*	*0.5*	*1.0*	*1.0*	*arizonica*
14923	*1.5*	*1.0*	*1.0*	*1.0*	*1.5*	*1.5*	*1.5*	*na*	*arizonica*
14924	*1.5*	*1.0*	*1.0*	*1.5*	*1.5*	*2.0*	*1.0*	*1.0*	*arizonica*
14925	*1.5*	*1.0*	*1.0*	*1.5*	*1.5*	*1.5*	*1.0*	*1.0*	*arizonica*
14917 group C	***1.0***	***1.5***	***2.0***	***1.0***	***2.0***	***1.0***	***1.0***	***2.0***	***arizonica***
14921	***1.0***	***1.5***	***2.0***	***1.0***	***3.0***	***1.0***	***1.0***	***2.0***	***arizonica***
14918	***1.0***	***2.0***	***2.0***	***1.0***	***3.0***	***1.0***	***1.0***	***2.0***	***arizonica***
14919	***1.0***	***2.0***	***2.0***	***1.0***	***3.0***	***1.0***	***1.0***	***2.0***	***arizonica***
14922	***1.0***	***2.0***	***2.0***	***1.0***	***3.0***	***1.0***	***1.0***	***2.0***	***arizonica***
14926	***1.0***	***2.0***	***2.0***	***1.0***	***3.0***	***1.0***	***1.0***	***2.0***	***arizonica***
14914 group D	1.0	2.0	2.0	1.0	3.0	1.0	1.5	2.0	*arizonica*
14927	1.0	2.0	1.0	1.0	2.0	1.0	1.5	3.0	*arizonica*
14929	1.0	3.0	1.0	1.0	3.0	1.0	1.5	3.0	*arizonica*
14928	1.0	2.0	2.0	1.0	3.0	1.0	1.5	3.0	*arizonica*
14930	1.0	2.0	2.0	1.5	3.0	1.0	1.5	3.0	*arizonica*
Average	1.11	1.72	1.58	1.11	2.44	1.06	1.17	1.91	
Sd	0.64	1.56	1.47	0.64	1.99	1.01	0.73	2.26	

Table 2) were closest to 1:1 ratios. Many of the hybrids had sites 177, 295, 370, and 1107 with 2:1 or 3:1 ratios. Of course, the parents were judged to be monomorphic based on visual inspection of the chromatograms at the 8 sites. There may have been some (~10% or less) polymorphism that was not evident on the chromatograms.

In contrast, sites 124, 313, 516, and 775 were generally inherited near the expected 1:1 ratio with average ratios of 1.11, 1.11, 1.06, and 1.17 (Table 2).

To further examine the variation among the hybrids, the nucleotides at each of the 8 sites were coded as the proportion of each base present (Table 3). Then, these data were subjected to Principal Coordinates Ordination (PCO). Factoring the similarity matrix resulted in three eigenroots that were larger than the average diagonal value. In addition, the eigenroots appeared to asymptote after the third eigenroot. These three eigenroots accounted for 79.45% of the variance among the hybrids and the theoretical hybrid.

Table 3. Variable sites in hybrids between *H. arizonica* (14854, 2000.75,0.2517) x *H. macrocarpa* (14858, 896.752) cross which differ at 8 sites. nrDNA (ITS) numbering is from the 5' end. Chromatogram peak heights are ratio of peaks expressed as arizonica bp + macrocarpa bp. (i.e. for 124, C:T 2:1 = 0.67,0.33; C:T 60:40 = 0.6,0.4, etc.). na = not available. petN sequence pattern (right-most column) shows the paternal (pollen) parent of the hybrid.

	124	177	295	313	370	516	775	1107	petN seq.
arizonica	C 1.0	C 1.0	T 1.0	C 1.0	G 1.0	C 1.0	G 1.0	A 1.0	*arizonica*
macrocarpa	T 1.0	G 1.0	C 1.0	T 1.0	T 1.0	T 1.0	A 1.0	G 1.0	*macrocarpa*
	C,T	C,G	T,C	C,T	G,T	C,T	G,A	A,G	
theoretical hybrid	0.5, 0.5	0.5, 0.5	0.5, 0.5	0.5, 0.5	0.5, 0.5	0.5, 0.5	0.5, 0.5	0.5, 0.5	
14914	0.5, 0.5	0.67,0.33	0.67, 0.33	0.5, 0.5	0.75, 0.25	0.5, 0.5	0.6, 0.4	0.67,0.33	*arizonica*
14915	0.5, 0.5	0.6, 0.4	0.67, 0.33	0.5, 0.5	0.75, 0.25	0.5, 0.5	0.5, 0.5	0.5, 0.5	*arizonica*
14916	0.6, 0.4	0.5, 0.5	0.55, 0.45	0.6, 0.4	0.6, 0.4	0.5, 0.5	0.5, 0.5	0.5, 0.5	*arizonica*
14917	0.5, 0.5	0.6, 0.4	0.67, 0.33	0.5, 0.5	0.67, 0.33	0.5, 0.5	0.5, 0.5	0.67,0.33	*arizonica*
14918	0.5, 0.5	0.67,0.33	0.67, 0.33	0.5, 0.5	0.75, 0.25	0.5, 0.5	0.5, 0.5	0.67,0.33	*arizonica*
14919	0.5, 0.5	0.67,0.33	0.67, 0.33	0.5, 0.5	0.75, 0.25	0.5, 0.5	0.5, 0.5	0.67,0.33	*arizonica*
14920	0.5, 0.5	0.67,0.33	0.5, 0.5	0.5, 0.5	0.67, 0.33	0.5, 0.5	0.5, 0.5	0.5, 0.5	*arizonica*
14921	0.5, 0.5	0.6, 0.4	0.67, 0.33	0.5, 0.5	0.75, 0.25	0.5, 0.5	0.5, 0.5	0.67,0.33	*arizonica*
14922	0.5, 0.5	0.67,0.33	0.67, 0.33	0.5, 0.5	0.75, 0.25	0.5, 0.5	0.5, 0.5	0.67,0.33	*arizonica*
14923	0.6, 0.4	0.5, 0.5	0.5, 0.5	0.5, 0.5	0.6, 0.4	0.6, 0.4	0.6, 0.4	na	*arizonica*
14924	0.6, 0.4	0.5, 0.5	0.5, 0.5	0.6, 0.4	0.6, 0.4	0.67,0.33	0.5, 0.5	0.5, 0.5	*arizonica*
14925	0.6, 0.4	0.5, 0.5	0.5, 0.5	0.6, 0.4	0.6, 0.4	0.6, 0.4	0.5, 0.5	0.5, 0.5	*arizonica*
14926	0.5, 0.5	0.67,0.33	0.67, 0.33	0.5, 0.5	0.75, 0.25	0.5, 0.5	0.5, 0.5	0.67,0.33	*arizonica*
14927	0.5, 0.5	0.67,0.33	0.5, 0.5	0.5, 0.5	0.67, 0.33	0.5, 0.5	0.6, 0.4	0.75,0.25	*arizonica*
14928	0.5, 0.5	0.67,0.33	0.67, 0.33	0.5, 0.5	0.75, 0.25	0.5, 0.5	0.6, 0.4	0.75,0.25	*arizonica*
14929	0.5, 0.5	0.75,0.25	0.5, 0.5	0.5, 0.5	0.75, 0.25	0.5, 0.5	0.6, 0.4	0.75,0.25	*arizonica*
14930	0.5, 0.5	0.67,0.33	0.67, 0.33	0.6, 0.4	0.75, 0.25	0.5, 0.5	0.6, 0.4	0.75,0.25	*arizonica*
14931	0.5, 0.5	0.6, 0.4	0.55, 0.45	0.5, 0.5	0.67, 0.33	0.5, 0.5	0.5, 0.5	0.6, 0.4	*arizonica*

PCO ordination revealed four groups (Fig. 2). Group A (15, 20, 31) appears most like the theoretical hybrid. Group B is the most distinct group. Group C is the most uniform and as hybrids 18, 19, 22, 16 had identical compositions. Group D is the least similar to the theoretical hybrid at the 8 polymorphic nrDNA sites.

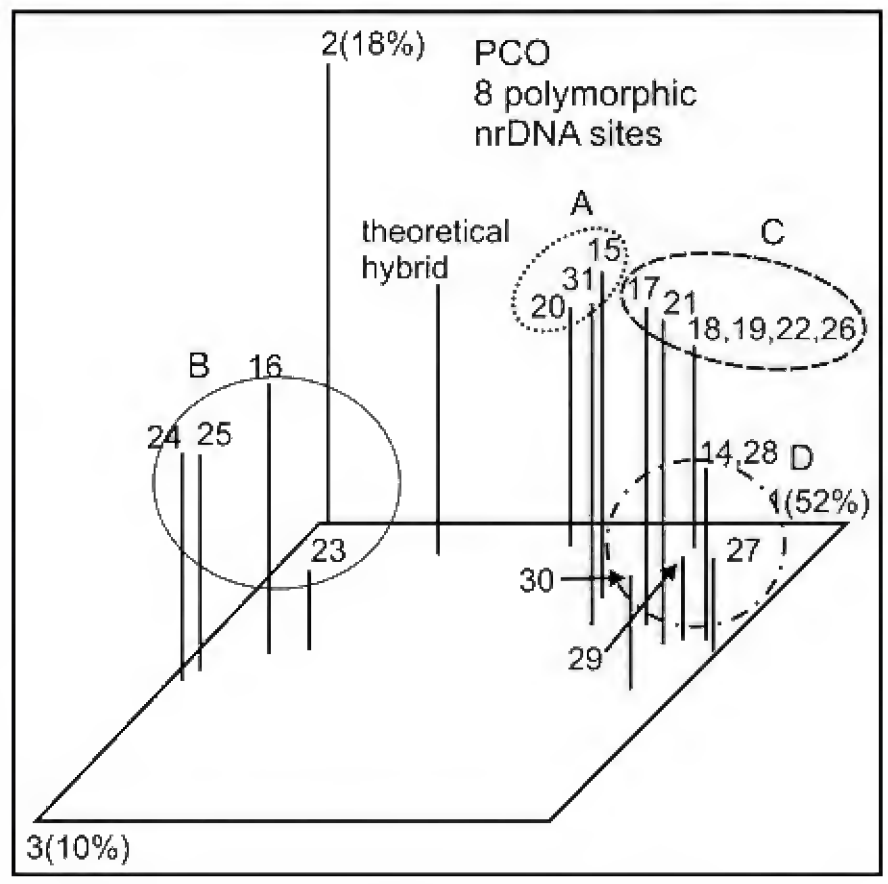

Fig. 2. PCO of hybrids based on relative peak heights. Numbers are the last two digits of the 5 digit plant number in Table 2.

It is interesting to note that if one denoted the *H. arizonica* parent's nrDNA as a1 (from one parent and a2 from the other parent), and likewise *H. macrocarpa* parent's nrDNA as m1, m2, then the nrDNA of the parents would be inherited in the progeny as: a1, m1 (25%), a1, m2 (25%), a2, m1 (25%), a2, m2 (25%). The four groups of nrDNA (Table 2) are: A: 3/18, 16.7%; B: 4/18, 22.2%; C: 6/18, 33.3%; D: 5/18, 27.7%). These data suggests the parents may have not been pure for their nrDNA.

To investigate the potential to utilize this nrDNA for the detection of hybrids, a PCO was performed including both parents and hybrids in
which data were coded as in Table 3. Factoring the similarity matrix resulted in four eigenroots that were larger than the average diagonal value. In addition, the eigenroots appeared to asymptote after the fourth eigenroot. The first three eigenroots accounted for 81.91% of the variance among the parents, hybrids and the theoretical hybrid. Ordination reveals (Fig. 3) the parents are well resolved with the hybrids in an intermediate position, but nearer to *H. arizonica*. This is quite similar to the U (or V) shaped pattern obtained by PCO using morphological data for artificial crosses in *Lepomis* (sunfish) (Adams, 1982, Fig. 4). Adams (1982, Fig. 9) also obtained a U (or V) shaped pattern in PCO based on 30 leaf terpenoids for *J. horizontalis*, *J. scopulorum* and putative hybrids. Thus, it appears, that nrDNA if appropriately coded can be utilized in multivariate methods for the detection of hybrids in Cupressaceae.

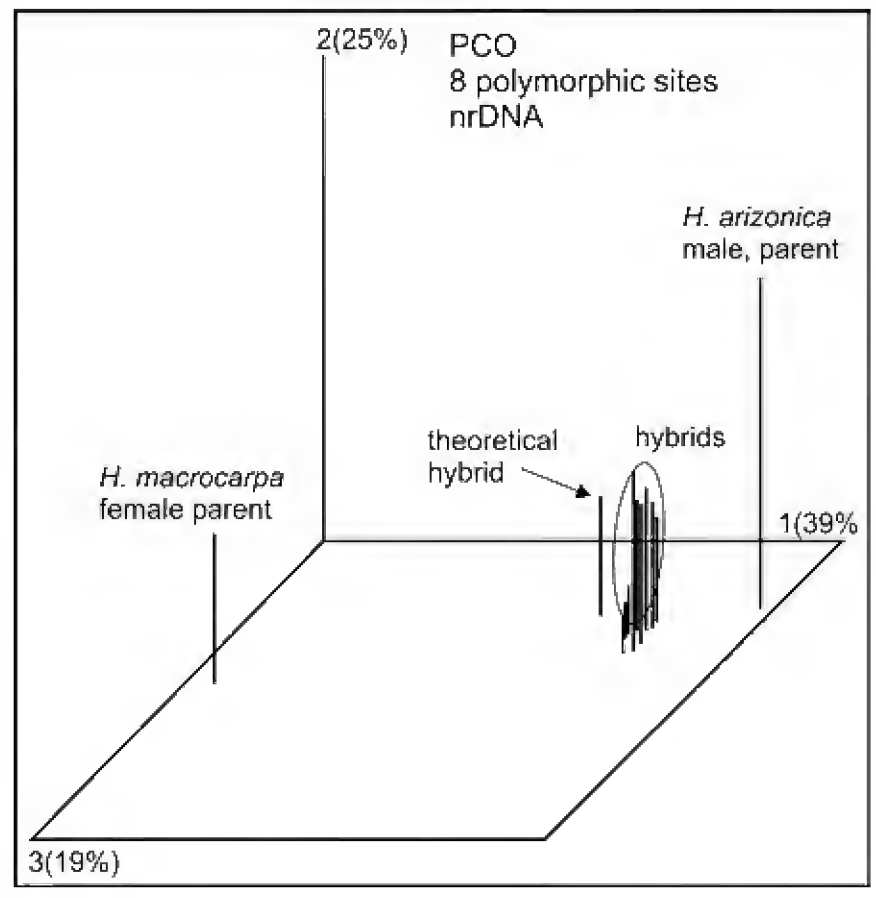

Fig. 3. PCO of parents, artificial hybrids, and the theoretical hybrid.

Analysis of petN-psbM (cp DNA) confirmed that all the hybrids inherited the cp genome from their pollen parent (*H. arizonica*).

The present study, shows a rather uniform inheritance of nrDNA in hybrids. The previous study on 7 putative backcross individuals of *Cryptomeria japonica* cultivars (Adams and Matsumoto, 2016) reported 3 of the 7 progeny had nrDNA very similar to parent (Haara x Kumo), and 4 progeny had nrDNA the same as recurrent parent Haara at positions 154 and 468 (Table 1). It appears that

backcrossing, some (4) of the backcrossed progeny reverted to the nrDNA of the recurrent parent, suggesting that nrDNA may be of somewhat limited value for the analysis of introgression.

The results of the *Cryptomeria japonica* backcrosses-study appear to parallel the Aguilar et al. (1999) study that found backcrosses showed homogenization of five of the polymorphic sites to the recurrent parent.

The present study on *Hesperocyparis* hybrids supports the use of nrDNA for the detection hybrids. But, due to concerted evolution (lineage sorting) in backcrosses (cf. *Cryptomeria japonica* and *Armeria* studies cited above), perhaps nrDNA analysis may underestimate the degree of introgression.

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